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Lizzi Vester

Lizzi Vester
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Titel: Recombinant MVA virus og anvendelse heraf

Ansøgere: Bavarian Nordic Research Institute A/S
Smedeland 26B, DK-2600 Glostrup

Opfindere:

Recombinant MVA virus, and use thereof

The present invention relates to recombinant MVA viruses containing and capable of expressing foreign genes which are inserted at the site of a naturally occuring deletion in the MVA genome, and the use of such recombinant MVA viruses for the production of polypeptides, e.g. antigens or therapeutic agents, or recombinant viruses for vaccines, or viral vectors for gene therapy.

Objects of the Invention

It is an object of the present invention to provide a recombinant MVA virus which can serve as an efficient and exeptionally safe expression vector.

Another object of the present invention is to provide a simple, efficient and safe method for the production of polypeptides, e.g. antigens or therapeutic agents, and recombinant viruses for vaccines or viral vectors for gene therapy.

Still another object of the the present invention is to provide an expression system based on a recombinant MVA virus according to the invention capable of expressing T7 RNA polymerase, and methods for the production of polypeptides, e.g. antigens or therapeutic agents, and for generating recombinant viruses for vaccines or viral vectors for gene therapy, based on this expression system.

Background of the Invention

Vaccinia viruses belong to the family of proviruses.
Certain strains of vaccinia viruses have been used for many years as live vaccine to immunize against smallpox, for example the Elstree strain of the Lister Institute in the UK. Because of the complications which may derive from the vaccination (Schär, Zeitschr. für Präventivmedizin 18, 41-44 [1973]), and since the declaration in 1980 by the WHO that smallpox had been eradicated nowadays only people at high risk are vaccinated against smallpox.

Vaccinia viruses have also been used as vectors for foreign antigens (Smith et al., Biotechnology and Genetic Engineering Reviews 2, 383-407 [1984]). This entails DNA sequences (genes) which code for foreign antigens being introduced, with the aid of DNA recombination techniques, into the genome of the vaccinia viruses. If the gene is integrated at a site in the viral DNA which is non-essential for the life cycle of the virus, it is possible for the newly produced recombinant vaccinia virus to be infectious, that is to say able to infect foreign cells and thus to express the integrated DNA sequence (EP Patent Applications No. 83, 286 and No. 110, 385). The recombinant vaccinia viruses prepared in this way can be used, on the one hand, as live vaccines for the prophylaxis of infections, on the other hand, for the preparation of heterologous proteins in eukaryotic cells. Recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase gene allowed the establishment of widely applicable expression systems for the synthesis of recombinant proteins in mammalian cells (Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W.A., and Fuerst T.R. (1990) Nature 348, 91-92.). In all protocols, recombinant gene expression relies on the synthesis of the T7 RNA polymerase in the cytoplasm of eukaryotic cells. Most popular became a protocol for transient-expression (Fuerst, T.R., Niles, E.G., Studier, F.W. and Moss, B. (1986) Proc. Natl. Acad. Sci. USA 83, 8122-8126 and US patent application 7.648.971). First, a foreign gene of interest is inserted into a plasmid under the control of the T7 RNA polymerase promoter. In the following, this plasmid is introduced into the cytoplasm of cells infected with a recombinant vaccinia virus producing T7 RNA polymerase using standard transfection procedures.

This transfection protocol is simple because no new recombinant viruses need to be made and very efficient with greater than 80% of the cells expressing the gene of interest (Elroy-Stein, O. and Moss, B. (1990) Proc. Natl. Acad. Sci. USA 87, 6743-6747. The advantage of the vaccinia virus/T7 RNA polymerase hybrid system over other transient expression systems is very likely its independence on the transport of plasmids to the cellular nucleus. In the past, the system has been extremely useful for analytical purposes in virology and cell biology (Buonocore, L. and Rose, J.K. (1990) Nature 345, 625-628, Pattnaik, A.K. and Wertz, G.W. (1991) Proc Natl. Acad. Sci. USA 88, 1379-1383, Karschin, A., Aiyar, J., Gouin, A., Davidson, N. and Lester, H.A. (1991) FEBS Lett. 278, 229-233, Ho, B.Y., Karschin, A., Raymond, J., Branchek, T., Lester, H.A. and Davidson, N. (1992) FEBS Lett. 301, 303-306, Buchholz, C.J., Retzler C., Homann, H.E., and Neubert, W.J. (1994) Virology 204, 770-776).

However, vaccinia virus is infectious for humans and its use as expression vector in the laboratory has been affected by safety concerns and regulations. Furthermore, possible future applications of the vaccinia virus/T7 RNA polymerase hybrid system, as e.g. to generate recombinant proteins or recombinant viral particles for novel therapeutic or prophylactic approaches in humans, are hindered by the productive replication of the recombinant vaccinia vector. Most of the recombinant vaccinia viruses described in the literature are based on the WR strain. On the other hand, it is known that this strain has a high neurovirulence and is thus poorly suited for use in humans and animals (Morita et al., Vaccine 5, 65-70 [1987]).

However, strains of viruses specially cultured to avoid undesired side effects have been known for a long time. Thus, it has been possible, by serial passages of the Ankara strain of vaccinia virus (CVA) on chicken embryo fibroblasts, to culture a modified vaccinia virus Ankara (MVA) (Swiss Patent No. 568, 392). The MVA virus has been analysed to determine alterations in the genome relative to the wild type CVA strain. Six major deletions (deletion I, II, III, IV, V, and VI) has been identified (Meyer, H., Sutter, G. and Mayr A. (1991) J. Gen. Virol. 72, 1031-1038). This modified vaccinia virus Ankara has only low virulence, that is to say it is followed by no side effects when used for vaccination. Hence it is particularly suitable for the initial vaccination of immunocompromised subjects. The excellent properties of the MVA strain have been demonstrated in a large number of clinical trials (Mayr et al., Zbl. Bakt. Hyg. I, Abt. Org. B 167, 375-390 [1987], Stickl et al., Dtsch. med. Wschr. 99, 2386-2392 [1974]).
Recently, a novel vaccinia vector system was established on the basis of the host range restricted and highly attenuated MVA virus, having foreign DNA sequences inserted at the site of deletion III within the MVA genome or within the TK gene (Sutter, G. and Moss, B. (1995) Dev. Biol. Stand. Basel, Karger 84, 195-200 and US patent 5.185.146). Derived by longterm serial passage in chicken embryo fibroblasts, MVA lost its capacity to grow productively in human and most other mammalian cells (Meyer, H., Sutter, G. and Mayr A. (1991) J. Gen. Virol. 72, 1031-1038 and Sutter et al., J. Virol., Vol. 68, No.7, 4109-4116 (1994)). Viral replication in human cells is blocked late in infection preventing the assembly to mature infectious virions. Nevertheless, MVA is able to express viral and recombinant genes at high levels even in non-permissive cells and can serve as an efficient and exceptionally safe expression vector (Sutter, G. and Moss, B. (1992) Proc. Natl. Acad. Sci. USA 89, 10847-10851).

To further exploit the use of MVA a novel possible way to introduce foreign genes by DNA recombination into the MVA strain of vaccinia virus has been sought. Since the intention was not to alter the genome of the MVA virus, it was necessary to use a method which complied with this requirement. According to the present invention a foreign DNA sequence was recombined into the viral DNA precisely at the site of a naturally occuring deletion in the MVA genome.

Summary of the Invention

The present invention thus, inter alia, comprises the following, alone or in combination:

A recombinant MVA virus containing and capable of expressing a foreign gene characterized in that the foreign gene is inserted at the site of a naturally occuring deletion within the MVA genome;

such a recombinant MVA wherein the foreign gene is inserted at the site of deletion II within the MVA genome;

such a recombinant MVA virus wherein the foreign gene codes for an antigenic determinant e.g. from a pathogenic virus, bacteria, parasite, or from a tumor cell;

such a recombinant MVA virus wherein the foreign gene codes for an antigenic determinant from Plasmodium Falciparum, Mycobacteria, Herpes virus, influenza virus, hepatitis, or human immunodeficiency viruses;

such a recombinant MVA virus wherein the foreign gene codes for a heterologous polypeptide e.g. a therapeutic agent;

such a recombinant MVA virus wherein the foreign gene codes for T7 RNA polymerase;

a recombinant MVA virus as any above wherein the foreign gene is under transcriptional control of the vaccinia virus early/late promoter P7.5;

a vaccine containing a recombinant MVA virus as any above wherein the foreign gene codes for an antigenic determinant e.g. from a pathogenic virus, bacteria, parasite, or from a tumor cell;

a method for the production of a polypeptide characterized in that a culture of eukaryotic cells is infected with a recombinant MVA virus as any above, the infected cells is cultured under suitable conditons to allow expression of the foreign gene, and the expressed product is isolated from the cell culture;

a method for the production of recombinant MVA particles characterized in that CEF cells infected with a recombinant MVA virus as any above is cultured under suitable conditions, and the viral particles is isolated from the cell culture;

an expression system comprising a recombinant MVA virus as any above wherein the foreign gene codes for T7 RNA polymerase and, one or more expression vectors containing one or more genes under transriptional control of a T7 RNA polymerase promoter;

a method for the production of a polypeptide characterized in that eucaryotic cells transformed with such an expression system, is cultured under suitable conditons to allow expression of the foreign gene, and the expressed product is isolated from the cell culture;

an expression system comprising a recombinant MVA virus as any above wherein the foreign gene codes for T7 RNA polymerase, and an expression vector carrying a construct comprising all of or a part of a viral genome under transriptional control of the T7 RNA polymerase promoter;

a method for the production of viral particles characterized in that eukaryotic cells transformed with such an expression system, is cultured under suitable conditions, and the viral particles is isolated from the cell culture;

expression system comprising a recombinant MVA virus as any above wherein the foreign gene codes for T7 RNA polymerase, and one or more expression vectors containing the gag, pol, and env genes of a retrovirus under transriptional control of the T7 RNA polymerase promoter, and an expression vector containing a retroviral vector construct comprising a provirus wherein the gag, pol and env genes has been replaced by one or more foreign genes;

a method for the production of retroviral vectors characterized in that eukaryotic cells transformed with such an expression system, is cultured under suitable conditions, and the retroviral vectors is isolated from the cell culture.

The term "gene" means any DNA sequence which codes for a protein or peptide.

The present Invention

Modified vaccinia virus Ankara (MVA), a host range restricted and highly attenuated vaccinia virus strain, is unable to multiply in human and most other mammalian cell lines tested. But since viral gene expression is unimpaired in non-permissive cells the recombinant MVA viruses according to the invention may be used as exceptionally safe and efficient expression vectors.

The present invention thus relates to recombinant MVA vaccinia viruses which contain a gene which codes for a foreign antigen, preferably of a pathogenic agent, and to vaccines which contain a virus of this type in a physiologically acceptable form. The invention also relates to methods for the preparation of such recombinant MVA vaccinia viruses or vaccines, and to the use of these vaccines for the prophylaxis of infections caused by such antigens or pathogenic agents.

The recombinant MVA vaccinia viruses according to the invention can also be used to prepare heterologous polypeptides in eukaryotic cells. This entails cells being infected with the recombinant vaccinia viruses. The gene which codes for the foreign polypeptide is expressed in the cells, and the expressed heterologous polypeptide is isolated. The methods to be used for the production of such heterologous polypeptides are generally

known to those skilled in the art (EP-A-206, 920 and EP-A- 205, 939). The polypeptides produced with the aid of the recombinant MVA viruses are, by reason of the special properties of the MVA viruses, more suitable for use as medicaments in humans and animals.

In one embodiment of the present invention we have constructed recombinant MVA viruses that allow expression of the bacteriophage T7 RNA polymerase gene under the control of the vaccinia virus early/late promoter P7.5. The usefulness of MVA-T7pol recombinant viruses as expression system was tested in transient transfection assays to induce expression of recombinant genes under the control of a T7 RNA ploymerase promoter. Using the E. coli chloramphenicol acetyltransferase (CAT) gene as a reporter gene we found that MVA-T7pol induced CAT gene expression as effectively as a vaccinia/T7pol recombinant virus derived from the replication-competent WR strain of vaccinia virus.
The MVA/T7 polymerase hybrid system according to the invention can thus be used as a simple, efficient and safe mammalian expression system for production of polypeptides in the absence of productive vaccinia virus replication.
This expression system can also be used for generating recombinant viral particles for vaccination or gene therapy by transformation of cell lines infected with recombinant MVA expressing T7 RNA polymerase, with DNA-constructs containing all or some of the genes, genome or recombinant genome nessesary for generating viral particles, under transcriptional control of a T7 RNA polymerase promoter.

The recombinant MVA virus expressing T7 RNA polymerase can for example be used to produce the proteins required for packaging retroviral vectors. To do this the gag,pol and env genes of a retrovirus (e.g. the Murine Leukemia Virus (MLV)) are placed under transcriptional control of a T7 RNA polymerase promoter in an expression vector (e.g. a plasmid). The expression vector is then introduced into cells infected with the recombinant MVA virus expressing T7 RNA polymerase, together with an expression vector carrying a retroviral vector construct comprising a provirus wherein the gag, pol and env genes has been replaced by one or more genes encoding foreign polypeptides (e.g. antigens, toxins or therapeutic polypeptides), possibly under transcriptional control of a T7 RNA polymerase promoter.

Another use of the recombinant MVA virus expressing T7 RNA polymerase is to generate recombinant proteins, non-infectious virus particles, or infectious mutant virus particles for the production of vaccines or therapeutics (Buchholz et al., Virology, 204, 770-776 (1994) and EP-B1-356695). To do this viral genes (e.g. the gag-pol and env genes of HIV-1) are placed under transcriptional control of the T7 promotor in an expression vector (e.g. plasmid or another recombinant MVA virus). This construct is then introduced into cells infected with the recombinant MVA virus expressing T7 RNA polymerase. The recombinant viral genes are transcribed with high efficiency, recombinant proteins are made in high amounts and can be purified. Additionally, expressed recombinant viral proteines (e.g. HIV-1 env, gag) may assemble to viral pseudo-particles that budd from the cells and can be isolated from the tissue culture medium. In another embodiment , viral proteins (from e.g. HIV Measles virus) expressed by the MVA T7 pol system may rescue an additionally introduced mutant virus (derived from e.g. HIV, Measles virus by overcoming a defect in attachment and infection, uncoating, nucleic acid replication, viral gene expression, assembly, budding or another step in viral multiplication to allow production and purification of the mentioned mutant virus.

The recombinant MVA vaccinia viruses can be prepared as set out hereinafter.

A DNA-construct which contains a DNA-sequence which codes for a foreign polypeptide flanked by the sequences flanking a naturally occuring deletion, e.g. deletion II, within the MVA genome, is introduced into cells infected with MVA, to allow homologous recombination.

Once the DNA-construct has been introduced into the eukaryotic cell and the foreign DNA has recombined with the viral DNA, it is possible to isolate the desired recombinant vaccinia virus in a manner known per se, preferably with the aid of a marker (compare Nakano et al., Proc. Natl. Acad. Sci. USA 79, 1593-1596 [1982], Franke et al., Mol. Cell. Biol. 1918-1924 [1985], Chakrabarti et al., Mol. Cell. Biol. 3403-3409 [1985], Fathi et al., Virology 97-105 [1986]).

The DNA-construct to be inserted can be linear or circular. A circular DNA is preferably used. It is particularly preferable to use a plasmid. The DNA-construct contains sequences flanking the left and the right side of a naturally occuring deletion, e.g. deletion

II, within the MVA genome (Altenburger, W., Suter, C.P. and Altenburger J. (1989) Arch. Virol. 105, 15-27).
The foreign DNA sequence is inserted between the sequences flanking the naturally occuring deletion. The foreign DNA sequence can be a gene coding for a therapeutic polypeptide, e.g. t-PA or interferon, or from a pathogenic agent. Pathogenic agents are to be understood to be viruses, bacteria and parasites which may cause a disease, as well as tumor cells which multiply unrestrictedly in an organism and may thus lead to pathological growths. Examples of such pathogenic agents are described in Davis, B.D. et al. , (Microbiology, 3rd ed., Harper International Edition). Preferred genes of pathogenic agents are those of the malaria parasite Plasmodium falciparum, of the tuberculosis-causing Mycobacteria, of herpes viruses and of human immunodificiency viruses, for example HIV I and HIV II.

In order for it to be possible for the foreign DNA sequence or the gene to be expressed, it is necessary for regulatory sequences, which are required for the transcription of the gene, to be present on the DNA. Such regulatory sequences (called promoters) are known to those skilled in the art, for example those of the vaccinia 11 kDa gene as are described in EP-A-198, 328, and those of the 7.5 kDa gene (EP-A-110, 385).

The DNA-construct can be introduced into the cells by transfection, for example by means of calcium phospate precipitation (Graham et al., Virol. 52, 456-467 [1973]; Wigler et al., Cell 777-785 [1979] by means of electroporation (Neumann et al., EMBO J. 1, 841-845 [1982]), by microinjection (Graessmann et al., Meth. Enzymology 101, 482-492 (1983)), by means of liposomes (Straubinger et al., Methods in Enzymology 101, 512-527 (1983)), by means of spheroplasts (Schaffner, Proc. Natl. Acad. Sci. USA 77, 2163-2167 (1980)) or by other methods known to those skilled in the art. Transfection by means of calcium phosphate precipitation is preferably used.

To prepare vaccines, the MVA vaccinia viruses according to the invention are converted into a physiologically acceptable form. This can be done based on the many years of experience in the preparation of vaccines used for vaccination against smallpox (Kaplan, Br. Med. Bull. 25, 131-135 [1969]). Typically, about 106-107 particles of the recombinant MVA are freeze-dried in 100ml of phosphate-buffered saline (PBS) in the presence of 2% peptone and 1% human albumin in an ampoule, preferably a glass ampoule. The

lyophilisate can contain extenders (such as mannitol, dextran, sugar, glycine, lactose or polyvinylpyrrolidone) or other aids (such as antioxidants, stabilizers, etc.) suitable for parenteral administration. The glass ampoule is then sealed and can be stored, preferably at temperatures below -20°C., for several months.

For vaccination the lyophilisate can be dissolved in 0.1 to 0.2 ml of aqueous solution, preferably physiological saline, and administered parenterally, for example by intradermal inoculation. The vaccine according to the invention is preferably injected intracutaneously. Slight swelling and redness, sometimes also itching, may be found at the injection site (Stickl et al., supra). The mode of administration, the dose and the number of administrations can be optimized by those skilled in the art in a known manner. It is expedient where appropriate to administer the vaccine several times over a lengthy period in order to obtain a high titre of antibodies against the foreign antigen.

The detailed example which follows is intended to contribute to a better understanding of the present invention. However, it is not intended to give the impression that the invention is confined to the subject-matter of the example.

Examples

1. Growing and purification of the viruses

1.1 Growing of the MVA virus

The MVA virus is a greatly attenuated vaccinia virus produced by serial passages of the original CVA strain on chicken embryo fibroblast (CEF) cultures. For a general rewiew of the history of the production, the properties and the use of the MVA strain of vaccinia, reference may be made to the summary published by Mayr et al. in Infection 3, 6-14 [1975]. Owing to the adaptation to CEF, growth of the MVA virus on other cell systems is greatly restricted, and plaque formation by the virus is now detectable only on CEF cells. In order not to alter the properties of the MVA virus it was normally grown on CEF cells, the host cell for which it had been adapted. To prepare the CEF cells, 11-days old embryos were isolated from incubated chicken eggs, the extremities were removed, and the embryos were cut into small pieces and slowly dissociated in a solution composed of 25% trypsin at room temperature for 2 hours. The resulting cell suspension was diluted with one volume of medium I (MEM Eagle, for example obtainable from Gibco, Basle, Switzerland; Order No. 072-1500) containing 5% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 mg/ml) and 2 mM glutamine and filtered through a cell screen (for example obtainable from Technomara AG, Zurich, Switzerland, Order No. Bellco 1985, 150 mesh), and the cells were sedimented by centrifugation at 2000 rpm in a bench centrifuge (Hermle KG, D-7209 Gosheim, FRG) at room temperature for 5 minutes. The cell sediment was taken up in 1/4 of the original volume of medium I, and the CEF cells obtained in this way were spread on cell culture dishes. They were left to grow in medium I in a CO_2 incubator at 37°C. for 1-2 days, depending on the desired cell density, and were used for infection either directly or after 1-2 further cell passages. A clear description of the preparation of primary cultures can be found in the book by R.I. Freshney, "Culture of animal cell", Alan R. Liss Verlag, New York [1983] Chapter 11, page 99 et seq.

MVA viruses were used for infection as follows. CEF cells were cultured in 175 cm^2 cell culture bottles. At 80-90% confluence, the medium was removed and the cells were incubated for one hour with an MVA virus suspension (0.01 infectious particles (=pfu) per cell, 0.01 ml/cm^2) in phosphate-buffered saline (PBS/Dulbecco, for example Animed AG, Muttenz, Switzerland, Order No. 23.100.10). Then medium I was added (0.2 ml/cm^2) and the bottles were incubated at 37°C. for 2-3 days until about 80% of the CEF cells had

lysed. The virus lysates were stored with the cells and medium, without treatment, in the cell culture bottles at -30°C. before further processing (purification etc.)

1.2 Purification of the viruses

The purification steps undertaken to obtain a virus preparation which was as pure as possible and free from components specific to the host cell were identical for the MVA and WR viruses (Joklik, Virology 18, 9-18 [1962], Zwartouw et al., J. gen. Microbiol. 29, 523-529 [1962]). The cell cultures which had been infected and then stored at -30°C. were thawed, the residual cells were shaken off or scraped off the plastic substrate, and cells and virus were removed from the medium by centrifugation (Sorvall centrigue, GSA rotor, 1 hour at 5000 rpm and 10° C.). The sediment, composed of viral and cell particles, was suspended once in PBS (10-20 times the volume of the sediment), and the suspension was centrifuged as above. The new sediment was suspended in 10 times the volume of RSB buffer (10mM Tris-HCl pH 8.0, 10mM KCl, 1mM MgCl2), and the suspension was briefly treated with ultrasound (Labsonic 1510 equipped with a 4 mm diameter tip, obtainable from Bender and Hobein, Zürich, Switzerland; 2x10 seconds at 60 watts and room temperature) in order to disintegrate remaining still intact cells and to liberate the virus particles from the cell membranes. The cell nuclei and the larger cell debris were removed in the subsequent brief centrifugation of the suspension (Sorvall GSA rotor obtainable from DuPont Co., D-6353 Bad Nauheim, FRG; 3 minutes at 3000 rpm and 10° C.). The sediment was once again suspended in RSB buffer, treated with ultrasound and centrifuged, as described above. The collected supernatants containing the free virus particles were combined and layered over a pad composed of 10 ml of 35% sucrose in 10mM Tris-HCl, pH 8.0, and centrifuged in a Kontron TST 28.38/17 rotor (Kontron Instrumente, Zurich, Switzerland; corresponds to a Beckman SW 27 rotor) for 90 minutes with 14,000 rpm at 10° C.). The supernatant was decanted, and the sediment containing the virus particles was taken up in 10ml of 10mM Tris-HCl, pH8.0, homogenized by brief treatment with ultrasound(2x10 seconds at room temperature, apparatus as described above), and applied to a stepped gradient for further purification. The steps of the gradient were each composed of 5 ml of sucrose in 10mM Tris-HCl, pH 8.0 (sucrose concentration steps: 20%, 25%, 30%, 35% and 40%). The gradient was centrifuged in a Kontron TST 28.38/17 rotor at 14,000 rpm 10° C. for 35 minutes. After this centrifugation, several discrete zones containing virus particles were visible in the region of the gradient between

30% and 40% sucrose. This region was siphoned off from the gradient (10 ml), the sucrose solution was diluted with PBS (20 ml) and the virus particles were sedimented therefrom by centrifugation (Kontron TST 28.38/17 rotor, 90 minutes at 14,000 rpm, 10° C.). the sediment, which now consisted mostly of pure virus particles, was taken up in PBS in such a way that the virus concentrations corresponded on average to 1-5 x 10^9 pfu/ml. The purified virus stock solution was used either directly or diluted with PBS for the subsequent experiments.

2. Construction and characterization of recombinant MVA viruses

2.1. Construction of vector plasmids

To allow the generation of recombinant MVA viruses novel vector plasmids had to be constructed. Insertion of foreign genes into the MVA genome was targeted precisely to the site of the naturally occuring deletion II in the MVA genome. Sequences of MVA DNA flanking the site of a 2500-bp deletion in the HindIII N fragment of the MVA genome (Altenburger, W., Suter, C.P. and Altenburger J Arch. Virol. 105, 15-27 (1989) were amplified by PCR and cloned into the multible cloning site of pUC18 (KpnI, SmaI, PstI and SphI). The primers for the left 600-bp DNA flank were 5'-CAG CAG GGT ACC CTC ATC GTA CAG GAC GTT CTC-3' and 5'-CAG CAG CCC GGG TAT TCG ATG ATT ATT TTT AAC AAA ATA ACA-3' (sites for restriction enzymes KpnI and SmaI are underlined). The primers for the right 550-bp DNA flank were 5'-CAG CAG CTG CAG GAA TCA TCC ATT CCA CTG AAT AGC-3' and 5'-CAG CAG GCA TGC CGA CGA ACA AGG AAC TGT AGC AGA-3' (sites for restriction enzymes PstI and SphI are underlined). Between these flanks of MVA DNA, a 3.2 kbp DNA fragment containing the *Escherichia coli lacZ* gene under control of the vaccinia virus late promoter P11 was inserted into the Bam*HI* site of the pUC multible cloning site to generate the plasmid pUCII LZ. In the following, a 3.1 kbp DNA fragment containing the entire gene of bacteriophage T7 RNA polymerase under control of the vaccinia virus early/late promoter P7.5 was excised with EcoRI from plasmid pTF7-3 (Fuerst, T.R., Niles, E.G., Studier, F.W. and Moss, B. (1986) P. N. A. S. USA 83, 8122-8126), modified by incubation with Klenow DNA polymerase to generate blunt ends, and cloned into a unique *SmaI* restriction site of pUCII LZ to make the plasmid transfer vector pUCII LZ T7pol (Figure 1). As transcriptional regulator for the expression of the T7 RNA polymerase gene the vaccinia virus early/late promoter P7.5 was chosen. Other than stronger vaccinia virus late promoters (e.g. P11) this promoter system allows expression of

recombinant genes immediately after the infection of target cells.The plasmid pUCII LZ T7pol that directs the insertion of the foreign genes into the site of deletion II of the MVA genome was used to generate the recombinant virus MVA T7pol.

2.2. Generation of MVA T7pol

CEF cells infected with MVA at a multiplicity of 0.05 $TCID_{50}$ per cell were transfected with plasmid as described previously (Sutter, G, Wyatt, L., Foley, P., Bennink, J. and Moss, B. (1994) Vaccine 12, 1032-1040). Recombinant MVA virus expressing the T7 RNA polymerase and co-expressing β-D-galactosidase (MVA P7.5-T7pol) was selected by five consecutive rounds of plaque purification in CEF cells stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (300 μg/ml). Subsequently, recombinant viruses were amplified by infection of CEF monolayers, and the DNA was analyzed by PCR to confirm genetic homogenity of the virus stock.

2.3. Characterization of gene expression of MVA T7pol

To monitor expression of T7 RNA polymerase by recombinant MVA T7pol [^{35}S]methionine -labeled polypeptides from virus infected tissue culture were analyzed. Monolayers of the monkey kidney cell line CV-1 grown in 12-well plates were infected with virus at a multiplicity of 20 $TCID_{50}$ per cell. At 3 to 5 hours after infection, the medium was removed, and the cultures were washed once with 1 ml of methionine free medium. To each well, 0.2 ml of methionine-free medium supplemented with 50 μCi of [^{35}S]methionine was added and incubated for 30 min at 37°C. Cytoplasmic extracts of infected cells were prepared by incubating each well in 0.2 ml of 0.5% Nonidet P-40 lysis buffer for 10 min at 37°C and samples were analyzed by SDS-PAGE. The metabolic labeling of the CV-1 cells with MVA T7pol revealed the synthesis of two additional polypeptides (i) a protein of about 116,000 Da representing the E. coli ß-galactosidase co-expressed to allow the screening for recombinant virus and (ii) a 98,000 Da protein with the expected size of the bacteriophage T7 RNA polymerase (Figure 2).

The large amount of β-galactocidase made by MVA T7pol is remarkable. The results from the *in vivo* labeling experiments demonstrate a very strong expression of the P11-*LacZ* gene construct when inserted into the MVA genome at the site of deletion II indicating that recombinant genes in MVA vector viruses might be expressed more efficiently when inserted into this locus of the MVA genome.

3. Transient expression system based on MVA T7pol

The usefulness of MVA-T7pol recombinant viruses as expression system in comparison to the WR-T7pol recombinant virus vTF7-3 (Fuerst et a. 1986) was tested by the co-transfection of DNA of a plasmid vector that is derived from pTM1 (Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W.A., and Fuerst T.R. (1990) Nature 348, 91-92) and contains (cloned into the NcoI and Bam*HI* sites of the pTM1 multible cloning site) the *E. coli* chloramphenicol acetyltransferase (CAT) gene under the control of a T7 RNA polymerase promoter (PT_7).

Transfected and infected CV-1 cells were suspended in 0.2 ml of 0.25 M Tris-HCl (pH 7.5). After three freeze-thaw cycles, the lysates were cleared by centrifugation, the protein content of the supernatants was determined, and samples containing 0.5, 0.25, 0.1 µg total protein were assayed for enzyme activity as described by Mackett, M., Smith, G.L. and Moss, B. (1984) J. Virol. 49, 857-864. After autoradiography, labeled spots were quantitated using the Fuji imaging analysis system.

The results in Figure 3 demonstrate that by using the highly attenuated vaccinia vector MVA it is possible to exploit the vaccinia virus-T7 RNA polymerase system as efficiently as by using a fully replication-competent vaccinia virus recombinant.

Claims:

1. A recombinant MVA virus containing and capable of expressing a foreign gene characterized in that the foreign gene is inserted at the site of a naturally occuring deletion within the MVA genome.

2. A recombinant MVA virus according to claim 1 characterized in that the foreign gene is inserted at the site of deletion II within the MVA genome.

3. A recombinant MVA virus according to claim 1 wherein the foreign gene codes for an antigenic determinant e.g. from a pathogenic virus, bacteria, parasite, or from a tumor cell.

4. A recombinant MVA virus according to claim 1 wherein the foreign gene codes for a heterologous polypeptide e.g. a therapeutic agent.

5. Vaccine containing a recombinant MVA virus according to claim 3.

6. A recombinant MVA virus according to claims 1 and 2 wherein the foreign gene codes for T7 RNA polymerase.

7. A recombinant MVA virus according to claims 1, 2 and 6 wherein the foreign gene is under transcriptional control of the vaccinia virus early/late promoter P7.5.

8. Expression system comprising a recombinant MVA virus according to claim 6 and one or more expression vectors containing one or more genes under transriptional control of a T7 RNA polymerase promoter.

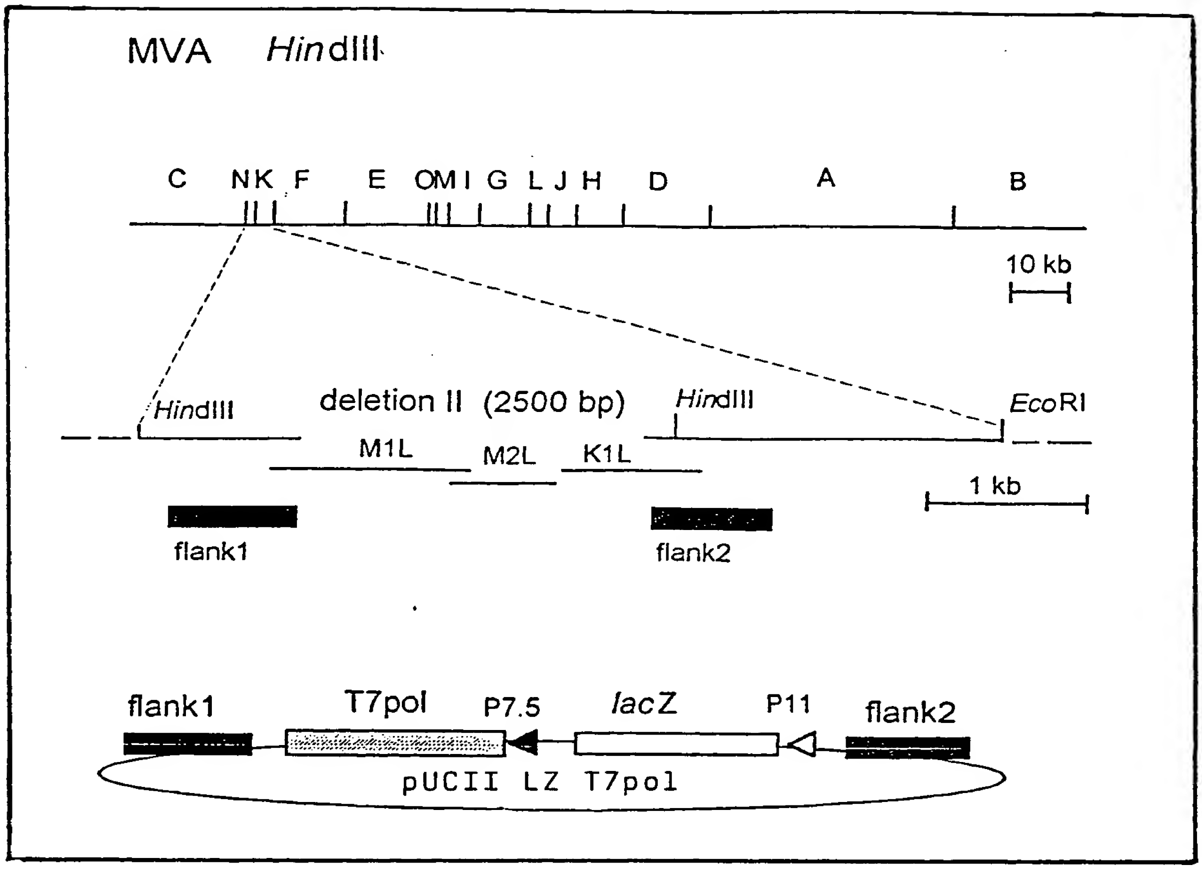
9. Expression system comprising a recombinant MVA virus according to claim 6 and an expression vector carrying a construct comprising all of or a part of a viral genome under transriptional control of a T7 RNA polymerase promoter.

10. Expression system comprising a recombinant MVA virus according to claim 6 and one or more expression vectors containing the gag, pol, and env genes of a retrovirus under transriptional control of a T7 RNA polymerase promoter, and an expression vector containing a retroviral vector construct comprising a provirus wherein the gag, pol and env genes has been replaced by one or more foreign genes.

Abstract

Recombinant MVA viruses containing and capable of expressing foreign genes which is inserted at the site of a naturally occuring deletion within the MVA genome, and the use of such recombinant MVA viruses for the production of polypeptides, e.g. antigenes or therapeutic agents, for the production of recombinant viruses for vaccines, or viral vectors for gene therapy.

FIGURE 1

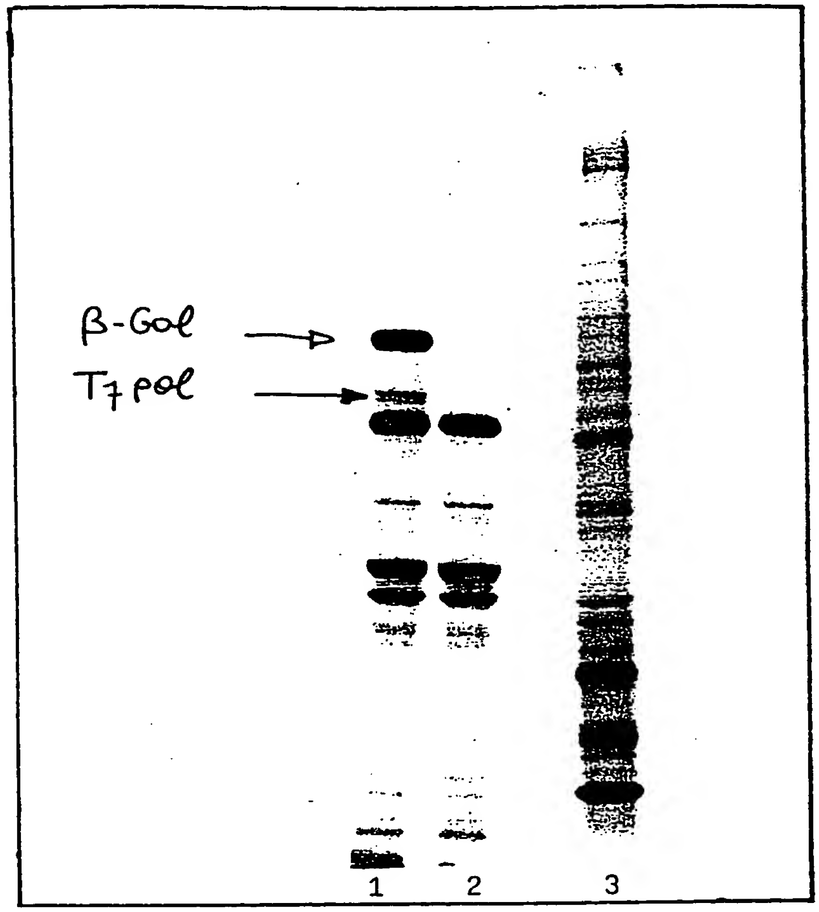


a) Schematic map of the MVA genome

b) Deletion II in the MVA genome

c) Plasmid vector pUCII LZ T7pol

FIGURE 2: Metabolic Labeling of Proteins

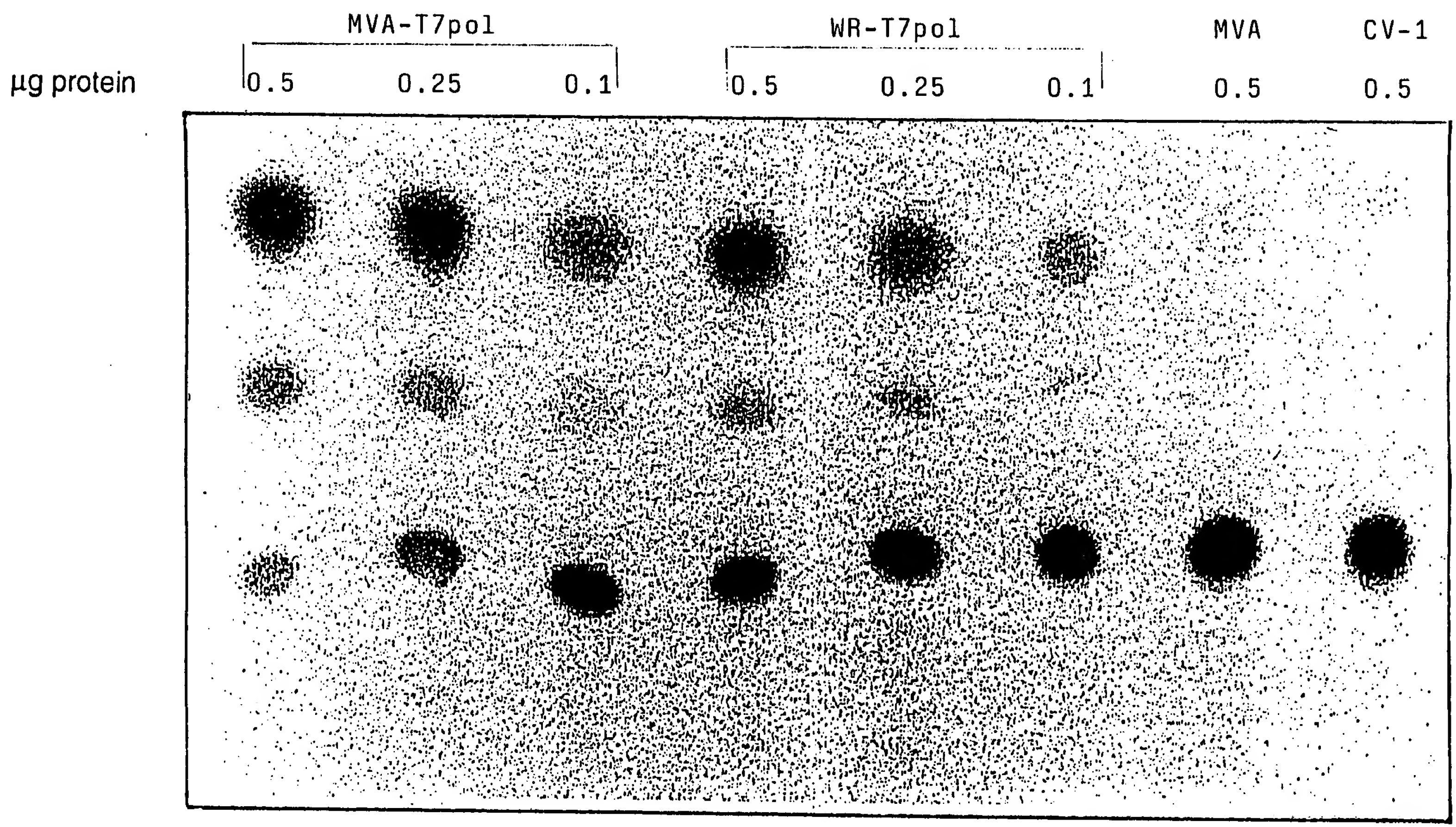


Lane 1: MVA T7pol

Lane 2: MVA

Lane 3: CV-1-cells

FIGURE 3 CAT Assay



Cat assay: CV-1 cells transfected with plasmid containing CAT gene under control of T7 RNA polymerase promotor and infected with MVA-T7pol/WR-T7pol. Lysates were tested for CAT activity.

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